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ORIGINAL ARTICLE

The effects of two nights of sleep deprivation with or without energy restriction on immune indices at rest and in response to cold exposure

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Abstract The purpose of the study was to determine the effects of two nights of sleep deprivation with or without energy restriction on immune indices at rest and in response to cold exposure. On three randomised occasions ten males slept normally [mean (SD): 436 (21) min night^{-1}; CON], were totally sleep-deprived (SDEP), or were totally sleep-deprived and 90% energy-restricted (SDEP + ER) for 53 h. After 53 h (1200 h) participants performed a seated cold air test (CAT) at 0.0°C until T_{re} decreased to 36.0°C. Circulating leucocyte counts, neutrophil degranulation, stress hormones and saliva secretory IgA (S-IgA) were determined at 0 h, 24 h, 48 h, pre-CAT, post-CAT, 1-h and 2-h post-CAT. One night on SDEP increased bacterially stimulated neutrophil degranulation (21%, $P < 0.05$), and two nights on SDEP and SDEP + ER increased S-IgA concentration (40 and 44%; $P < 0.01$). No other significant effects were observed for immuno-endocrine measures prior to CAT. CAT duration was not different between trials [mean (SD): 133 (53) min] and T_{re} decreased to 35.9 (0.3)°C. Modest whole-body cooling decreased circulating lymphocyte counts (25%; $P < 0.01$), S-IgA concentration (36%; $P < 0.01$) and secretion rate (24%; $P < 0.05$). A neutrophilia occurred post-CAT on CON and SDEP and 2-h post-CAT on SDEP + ER ($P < 0.01$). Modest whole-body cooling also decreased neutrophil degranulation on CON (22%) and SDEP (18%; $P < 0.05$). Plasma cortisol and norepinephrine increased post-CAT (31 and 346%, $P < 0.05$), but modest whole-body cooling did not alter plasma epinephrine. In conclusion, two nights of SDEP or SDEP + ER did not compromise resting immune indices. However, modest whole-body cooling (T_{re} 35.9°C) decreased circulating lymphocytes, neutrophil degranulation and S-IgA, but responses were not amplified by prior SDEP or SDEP + ER.

Keywords Nutrition · Hypothermia · Neutrophil · Lymphocyte · Saliva · S-IgA

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Introduction

Stressors including physical exertion, energy restriction, sleep deprivation, environmental extremes and anxiety are often encountered by those with extremely demanding occupations and pastimes such as military personnel, mountaineers and ultra-endurance athletes. These stressors may evoke suboptimal host defence which in-turn may increase the risk of illness, infection, decrease effective wound healing and increase the susceptibility to sepsis following injury in the field (Shephard et al. 1998).

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Stress-induced immune perturbations have been attributed to stimulation of the sympatheticoadrenal-medullary (SAM) and hypothalamic-pituitary adrenal (HPA) axis and subsequent increases in circulating immune-modulating stress hormones (e.g. catecholamines and cortisol) (Gleeson 2007). In addition, energy restriction may directly decrease immune function since nutrients are involved in immune cell proliferation, metabolism and antioxidant defences (Gleeson 2007).

It is therefore conceivable that impairment of immune responses may occur during activities involving a combination of stressors. For example, military field studies incorporating 5–7 days of heavy exertion, energy restriction, sleep deprivation and exposure to environmental extremes report decreased cellular, humoral and mucosal immunity and increased infection rates leading to course failure (Boyum et al. 1996; Martinez-Lopez et al. 1993; Tiollier et al. 2005). However, it is not possible to determine whether an individual stressor or a combination of a number of stressors is responsible for the decreased immune activity during these field studies due to lack of adequate research control. It is likely that the interaction of these stressors on immune function is complex further supporting the notion that investigations should identify both the independent and combined effects of stressors (e.g. sleep deprivation and energy restriction) on immune function. Such an experimental approach is important because these stressors may be experienced alone or in combination in the field setting and because the results from appropriately designed laboratory studies may provide important information about effective countermeasures.

Although heavy exertion and prolonged nutritional restriction are now widely recognised to decrease many aspects of immune function (Gleeson 2007; Laing et al. 2008b; Oliver et al. 2007), the influence of sleep deprivation and exposure to environmental extremes, particularly cold exposure, remain topics of debate (Costa et al. 2009; Walsh and Whitham 2006). A recent study showed that 30 h of total sleep deprivation did not alter leucocyte trafficking, neutrophil degranulation or saliva S-IgA responses either at rest or in response to exercise (Costa et al. 2009). However, longer periods of total sleep deprivation (≥48 h) have been associated with a leucocytosis (Dinges et al. 1994) and decreases in neutrophil and lymphocyte function (Moldofsky et al. 1989; Palmblad et al. 1976, 1979). Somewhat surprisingly, there is also limited and somewhat conflicting information about the effects of cold exposure on immune function (Walsh and Whitham 2006). Evidence to date indicates that a very mild decrease in body core temperature (T_{re} decrease ~0.5°C) during short (30 min) (Lackovic et al. 1988) or prolonged (2 h) (Brenner et al. 1999) cold air exposure can actually have immuno-stimulatory effects; yet little information is available from tightly controlled laboratory studies about the effects of a decrease in body core temperature ≥1.0°C on immune function. One study showed that severe hypothermia in patients during surgery (T_{re} decrease ~4.0°C) decreased aspects of neutrophil function, but lack of experimental control is a limitation in that study (Wenisch et al. 1996). It is conceivable that a continuum exists for the effects of body core cooling on immune function whereby very mild decreases in body core temperature have little or even stimulatory effects on immune function, but more severe decreases in body core temperature have depressive effects on immune function. It remains to be shown though whether immune function is decreased by a more modest reduction in body core temperature, as may be experienced by military personnel during training and operations in cold and wet conditions (e.g. T_{re} decrease 1–2°C).

With this information in mind, the purpose of the present study was to determine the effects of two nights of sleep deprivation with or without energy restriction on immune indices at rest and in response to cold exposure. We hypothesised that sleep deprivation and energy restriction would have additive detrimental effects on immune indices at rest. We also hypothesised that a modest reduction in body core temperature would decrease immune indices and that prior sleep deprivation and energy restriction would amplify this response.

Methods

Participants

Ten healthy, recreationally active males [mean (SD): age 25.0 (6.3) years; height 178 (4) cm; nude body mass (NBM) 79.4 (8.6) kg; body fat 17 (5) %; $\dot{V}O_{2max}$ 57.3 (8.4) ml kg^{-1} BM min^{-1}] volunteered to participate in the study. Participants gave written informed consent prior to the study, which received local ethics committee approval. Participants reported no symptoms of infection or illness 12 weeks prior and during the study, and no medications or supplements were taken in the 6 weeks prior or during the study. Participants were also asked to refrain from alcohol and caffeine for 72 h, and exercise for 24 h prior to the preliminary testing session and each experimental trial.

Preliminary testing

Prior to the main experimental trials, participants were transported to the laboratory at 0800 h after an overnight fast. Following bowel and bladder voiding, height and NBM (STW-150KE, Hampel Electronics, Zhonghe,

Taiwan) were measured. Body composition was determined by whole-body dual-energy X-ray absorptiometry (QDR1500, Hologic, MA, USA). Resting metabolic rate (RMR) was determined by indirect calorimetry (Compher et al. 2006) and adjusted by a general daily physical activity and diet-induced thermogenesis factor coefficient of 1.2 (Todorovic and Micklewright 2004). Participants were then familiarised with a 20-min cold air test (CAT), which was conducted in an environmental chamber (WIR Series, Design Environmental Ltd., Ebbw Vale, UK) set at 0.0°C and 40% relative humidity (RH).

After a 30-min rest period, maximal oxygen uptake ($\dot{V}O_{2max}$; Cortex Metalyser 3B, Biophysik, Leipzig, Germany) was estimated by means of a continuous incremental exercise test to volitional exhaustion (Oliver et al. 2007) on a motorised treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany). The treadmill walking speed [mean (SD): 6.0 (0.5) km h^{-1}] and gradient [mean (SD): 6.9 (2.0) %] that elicited 50% $\dot{V}O_{2max}$ for the participants' steady-state exercise was extrapolated and verified. In addition, each participant completed a time trial (TT) familiarisation. Blinded to the treadmill speed, participants controlled the speed of the motorised treadmill at 1% gradient, and were instructed to run 5 km as quickly as possible. Estimated energy expenditure for steady-state exercise and the TT was predicted by indirect calorimetry and combined with the adjusted RMR data to predict total daily energy expenditure for the experimental trials.

Experimental trials

Using a randomly assigned repeated measures design, participants completed three experimental trials separated by 12 days (Fig. 1). The three experimental trials included a control trial (CON), a sleep deprivation trial (SDEP) and a sleep deprivation and energy restriction trial (SDEP + ER). Participants were allowed normal nocturnal sleep on CON [mean (SD): 436 (21) min $night^{-1}$], but were totally deprived of sleep on SDEP and SDEP + ER for 53 h. Dietary intake was provided to meet predicted total daily energy requirements [mean (SD): 3,316 (505) kcal day^{-1}, 54 (2) % carbohydrate, 31 (2) % fat, 14 (2) % protein] on CON and SDEP, but was 90% restricted on SDEP + ER for 53 h. Water was provided throughout each experimental trial at 2-h intervals (divided equally over 16 h;

PRE-TRIAL DAY	**CONTROLLED PHYSICAL ACTIVITY, FOOD AND FLUID INTAKE**											**SLEEP**
DAY 1 0700 h awakening **CON** **SDEP** **SDEP+ER**	**SAMPLES**	**BREAKFAST**	**90 min WALK @ 50% VO_{2max}+ 5km TT**	**LUNCH**		**SNACK**		**DINNER**		**SNACK**		**SLEEP or SDEP**
DAY 2 0700 h awakening on CON	**SAMPLES**	**BREAKFAST**	**90 min WALK @ 50% VO_{2max}+ 5km TT**	**LUNCH**		**SNACK**		**DINNER**		**SNACK**		**SLEEP or SDEP**
	0800h	0845h	12-1445h	1500h		1730h		2015h		2215h		2300-0700h

DAY 3 0700 h awakening on CON	**SAMPLES**	**BREAKFAST**	**SAMPLES**	**CAT (53h)** T_{amb} 0.0°C + 40% RH	**T_{re}: 36.0°C SAMPLES**	**RE-WARM**	**SAMPLES**	**RE-FEED**	**SAMPLES**	**LEAVE**
	0800h	0845h	1145h	12-1600h	13-1600h	13-1800h	14-1700h	14-1700h	15-1800h	15-1800h

Fig. 1 Schematic of trial events. Control (CON): nocturnal sleep [mean (SD): 436 (21) min $night^{-1}$]; total sleep-deprivation (SDEP): 53-h total sleep-deprivation; total sleep deprivation and energy restriction (SDEP + ER): 53-h total sleep-deprivation and 90% energy restriction. Samples: nude body mass, venepuncture blood and unstimulated whole saliva. *TT* time trial; *CAT* cold air test

0700–2300 h), equivalent to 35 ml kg^{-1} BM day^{-1} [mean (SD): 2,785 (300) ml day^{-1}; Todorovic and Micklewright 2004], with estimated fluid losses during exercise added to this total. Daily physical activity was monitored by electronic pedometer (Digi-walker SW-200, Yamax, Tokyo, Japan), and sleep-wake cycles were monitored by an accelerometer (GT1 M, ActiGraph LLC, Florida, USA). Participants remained in the laboratory living quarters for the entire experimental period during all trials and were supervised at all times. NBM measurements were taken at 0 h, 24 h, 48 h, pre-CAT, post-CAT, 1-h and 2-h post-CAT.

Experimental procedures

The day prior to each experimental trial, to standardise nutrition, hydration and sleep-wake cycles, participants were provided with their predicted total daily energy and fluid requirements, and nocturnal sleep duration was monitored. After awakening at 0700 h on day 1, participants were transported to the laboratory for each experimental trial. Participants were requested to empty their bladder and bowels prior to 0-h measurements. Urine specific gravity, determined by a handheld refractometer (Atago Uricon-Ne, NSG Precision Cells, New York, USA), was 1.015 (SD 0.005) g l^{-1} on arrival at the laboratory, indicating euhydration (Armstrong et al. 1994). At 1200 h on days 1 and 2, participants performed 90-min steady-state exercise, followed by a 15-min seated rest period and then a 5-km TT. Both exercise bouts were performed in an air-conditioned laboratory [mean (SD): 20 (1) °C, 59 (7) % RH] with one fan placed 1 m from the treadmill set to a speed of 2.3 m s^{-1}. Each TT was performed under standardised conditions in a quiet laboratory, with only information about distance completed provided to the participants. Water was consumed ad libitum during steady-state exercise in all trials, while no fluids were allowed during the TT.

At 1200 h on day 3, participants performed a CAT conducted in an environmental chamber. Prior to the CAT, participants fitted a HR monitor, and a thermocouple was inserted 12 cm beyond the external anal sphincter (Grant REC soft insertion probe thermocouple; Grant 2020 Squirrel data logger, Shepreth, UK) to monitor rectal temperature (T_{re}). Participants performed the CAT clothed in only athletic shorts, socks and training shoes. Ambient conditions throughout the CAT were regulated at 0.0°C, 40% RH and 0.2 m s^{-1} wind velocity. T_{re} was recorded immediately prior to and every 5 min during the CAT, whereas HR, ratings of McGinnis 13-point thermal comfort (Hollies and Goldman 1977) and 10-point pain sensation (Chen et al. 1998) were recorded immediately prior to and every 10 min during the CAT.

Participants assumed a standardised seated position on a steel framed wooden laboratory chair and were instructed to minimise any movements, including behavioural thermoregulation (e.g. rubbing and fidgeting) during the CAT. Participants remained in the environmental chamber until their T_{re} reached 36.0°C or they achieved the cut-off criteria of 4-h exposure. As a safety precaution, recovery T_{re} was monitored every 5 min during the first hour after the CAT. Throughout this recovery period, participants remained seated and were wrapped in a blanket. One hour after the CAT, participants were allowed to shower, re-clothe and consume a standard meal (1,230 kcal, 58% carbohydrate, 28% fat and 14% protein). After the experimental trial, participants were transported to their homes.

Sample collection and analytical methods

Saliva

Unstimulated whole saliva samples were collected by dribble for 4 min into pre-weighed universal tubes (HR 120-EC, A & D instruments, Tokyo, Japan) at 0 h, 24 h, 48 h, pre-CAT, post-CAT, 1-h and 2-h post-CAT. Saliva flow rate was determined by weighing the tubes after collection and dividing the volume of saliva by the collection time as previously described (Oliver et al. 2007). Aliquots of saliva were pipetted into eppendorfs and stored frozen at −80°C prior to analysis. After thawing, saliva secretory IgA (S-IgA) concentration was determined by polymeric-IgA-directed ELISA (Immundiagnostik, Bensheim, Germany). S-IgA secretion rate was calculated by multiplying the saliva flow rate by S-IgA concentration. The intra-assay coefficient of variation for S-IgA concentration was 2.4%.

Blood

Whole blood samples were collected, without venestasis by venepuncture from an antecubital vein at 0 h, 24 h, 48 h, pre-CAT, post-CAT, 1-h and 2-h post-CAT into two K_3EDTA and two lithium heparin vacutainer tubes (Becton Dickinson, Oxford, UK). Haematocrit was determined by capillary method in triplicate by using lithium heparin blood samples and a micro-haematocrit reader (Hawksley & Sons Ltd., Lancing, UK). One K_3EDTA vacutainer tube was stored at room temperature prior to haematological analysis within 6 h of collection. Haematological analysis, which included haemoglobin concentration, circulating total and differential leucocyte counts, was performed using an automated cell counter (Gen-S, Beckman Coulter, High Wycombe, UK). Remaining blood samples were centrifuged at 1,500*g* for 10 min at 4°C within 15 min of sample collection. Lithium heparin and K_3EDTA plasma was

aspirated into eppendorfs and stored at −80°C for further analysis. All blood-borne parameters were corrected for changes in plasma volume (Dill and Costill 1974).

For each blood sample taken, 1 ml of lithium heparin blood was added to 50 µl of 10 mg ml^{-1} bacterial stimulant (Sigma, Poole, UK) within 5 min of collection and gently vortex mixed. Samples were immediately placed in a water bath at 37°C for 60 min as previously reported (Laing et al. 2008a). Samples were inverted at 30 min. After 60 min, samples were centrifuged at 5,000*g* for 2 min. Supernatant was then aspirated into eppendorfs and stored at −80°C for further analysis. Plasma elastase concentration, a marker of neutrophil degranulation, was measured in unstimulated and bacterially stimulated lithium heparin plasma by ELISA (Biovendor Laboratory Medicine, Modrice, Czech Republic). All samples for elastase release were run on the same day, with standards and controls for each plate. The intra-assay coefficient of variation for elastase concentration was 2.8%.

Plasma glucose was determined using lithium heparin plasma by a spectrophotometric method (Randox, County Antrim, UK). Aliquots of lithium heparin plasma were also used to determine plasma cortisol concentration by ELISA (DRG Diagnostics, Marburg, Germany). All cortisol samples analysed were run on the same day, with standards and controls on each plate. The intra-assay coefficient of variation was 3.4% for plasma glucose and cortisol. Aliquots of K_3EDTA plasma were used for the determination of plasma epinephrine and norepinephrine concentrations using high-pressure liquid chromatography (Clinrep, Recipe Chemicals, Munich, Germany), as previously described (Laing et al. 2008a). The intra-assay coefficient of variation for plasma epinephrine and norepinephrine was 5.0 and 8.0%, respectively.

Statistical analysis

Data in text are presented as mean value and standard deviation (SD), whereas data in tables and figures are presented as mean value and standard error of the mean (SEM) unless otherwise indicated. The data were examined using a two-way repeated measures analysis of variance (ANOVA) design, except for sleep quantity, TT performance and time to T_{re} 36.0°C during the CAT which was examined using a one-way ANOVA. Assumptions of homogeneity and sphericity were checked, and where appropriate, adjustment to the degrees of freedom was made using the Greenhouse–Geisser correction method. Significant main effects were analysed using post-hoc Tukey's HSD test. The required sample size was estimated to be between four and ten participants (http://www.dssresearch.com/toolkit/sscalc) using previous data examining the effects of combined stressors on selected immune indices (Laing et al. 2008a, b; Oliver et al. 2007). Alpha and beta levels were set at 0.05 and 0.8, respectively, both of which are standard estimates. The acceptance level of significance was set at $P < 0.05$.

Results

Nude body mass, daily physical activity and sleep quantity

A trial × time interaction was observed for NBM [$F(10,90) = 35.3$, $P < 0.01$; Table 1] and daily physical activity [$F(6,54) = 7.3$, $P < 0.01$; Table 1]. NBM gradually decreased throughout SDEP + ER ($P < 0.01$ vs. 0 h), whereas a decrease in NBM was only observed pre- to post-CAT on CON and SDEP ($P < 0.01$ vs. pre-CAT). Total pedometer counts were significantly higher on SDEP and SDEP + ER compared with CON ($P < 0.01$). No significant difference in sleep quantity was observed the night prior to each experimental trial and during CON (Table 1).

Time trial performance

No difference in TT performance was observed between trials on day 1 [CON: 23 (6) min; SDEP: 24 (4) min;

Table 1 Nude body mass changes, daily physical activity and sleep quantity ($n = 10$) during a 53-h period of normal sleep (CON), total sleep-deprivation (SDEP), and total sleep-deprivation and 90% energy-restriction (SDEP + ER) prior to a cold air test (CAT)

	CON	SDEP	SDEP + ER
Nude body mass (%)			
0 h	78.8 (8.6) kg	78.7 (8.6) kg	79.0 (8.3) kg
24 h	−0.4 (0.4)	−0.2 (0.6)	−2.1 (0.7)$^{\#,a,b}$
48 h	−0.6 (0.5)$^{\#}$	−0.7 (0.6)$^{\#}$	−3.6 (0.8)$^{\#,a,b}$
Pre-CAT	0.0 (0.5)	−0.4 (0.6)	−3.8 (0.8)$^{\#,a,b}$
Post-CAT	−1.1 (0.7)$^{\#,*}$	−1.4 (0.7)$^{\#*}$	−4.4 (0.9)$^{\#,*,a,b}$
Physical activity (steps day^{-1})			
Day-1 total	21,384 (2,236)	23,188 (4,120)	23,351 (3,049)
Day-2 total	20,425 (1,946)	24,769 (3,491)^{a}	25,043 (4,058)^{a}
Sleep quantity (min $night^{-1}$)			
Pre-trial	437 (45)	446 (21)	449 (20)
Night one	432 (25)	0	0
Night two	440 (16)	0	0

Mean (SD)

$^{\#}$ $P < 0.05$ versus 0 h

* $P < 0.05$ versus pre-CAT

^{a} $P < 0.05$ versus CON

^{b} $P < 0.05$ versus SDEP

SDEP + ER: 25 (7) min], whereas TT performance on day 2 (after 29 h of the intervention) was significantly poorer on SDEP + ER [32 (11) min] compared with CON [23 (5) min] and SDEP [24 (5) min; $P < 0.05$].

Cold air test measurements

There was no significant difference in pre-CAT T_{re} between trials [CON: 37.18 (0.19) °C; SDEP: 37.12 (0.15) °C; SDEP + ER: 37.03 (0.14) °C]. No significant difference in time to T_{re} 36.0°C was observed between trials [CON: 116 (38) min; SDEP: 153 (65) min; SDEP + ER: 130 (51) min] and T_{re} decreased to 35.9 (0.3) °C 20-min post-CAT. There were no trial × time interactions for HR, T_{re}, thermal comfort and pain sensation scales during the CAT, and T_{re} during the 1-h recovery after the CAT.

Plasma volume changes

A trial × time interaction was observed for plasma volume change [$F(4,36) = 2.6$; $P < 0.05$]. Compared with 0 h, a significant increase in plasma volume was observed at 24 h on CON (4.0%; $P < 0.05$), and at 24 h (5.7%) and 48 h (9.3%) on SDEP ($P < 0.01$). Plasma volume change was significantly greater on SDEP at 48 h compared with CON and SDEP + ER ($P < 0.01$). The CAT also elicited a change in plasma volume [main effect of time; $F(3,27) = 85.1$, $P < 0.01$] whereby decreases in plasma volume were observed post-CAT (13.5%), 1 h (9.1%) and 2-h post-CAT (4.7%) compared with pre-CAT values ($P < 0.01$).

Circulating leucocyte, neutrophil and lymphocyte counts

A trial × time interaction was observed for circulating leucocyte [$F(12,108) = 2.8$, $P < 0.01$; Fig. 2] and neutrophil counts [$F(12,108) = 3.0$, $P < 0.01$; Fig. 2], whereas a main effect of time was observed for circulating lymphocyte counts [$F(6,54) = 19.4$, $P < 0.01$; Fig. 2]. The CAT elicited a significant leucocytosis and neutrophilia ($P < 0.01$) on CON and SDEP which remained significantly elevated during recovery ($P < 0.01$). In contrast, a significant neutrophilia was observed 2-h post-CAT on SDEP + ER ($P < 0.05$). Circulating leucocyte and neutrophil counts on SDEP + ER were significantly lower post-CAT and 1-h post-CAT compared with SDEP ($P < 0.01$), and post-CAT ($P < 0.05$), 1-h ($P < 0.05$) and 2-h post-CAT ($P < 0.01$) compared with CON. The CAT also induced a significant lymphopenia 1-h and 2-h post-CAT ($P < 0.01$ vs. pre-CAT).

Neutrophil degranulation

No main effects were observed for unstimulated plasma elastase concentration. A trial × time interaction was observed for neutrophil degranulation (bacterially stimulated elastase release per neutrophil; $F(12,108) = 2.7$, $P < 0.01$), whereas a main effect of time was observed for bacterially stimulated plasma elastase concentration [$F(6,54) = 17.3$, $P < 0.01$]. SDEP increased neutrophil degranulation at 24 h only (21%; $P < 0.05$) compared with 0 h. Bacterially stimulated plasma elastase concentration increased post-CAT (40%; $P < 0.01$ vs. pre-CAT) and remained significantly elevated during recovery ($P < 0.01$ vs. pre-CAT). The CAT also induced significant decreases in neutrophil degranulation 1-h (22%; $P < 0.01$) and 2-h post-CAT (19%; $P < 0.05$) on CON, and post-CAT (18%) and 1-h post-CAT (18%) on SDEP ($P < 0.05$; Fig. 3). Compared with SDEP + ER, neutrophil degranulation was significantly lower 1-h ($P < 0.05$) and 2-h post-CAT ($P < 0.01$) on CON ($P < 0.01$), and 1-h post-CAT on SDEP ($P < 0.05$).

Saliva secretory IgA (S-IgA)

A trial × time interaction was observed for saliva S-IgA concentration [$F(12,108) = 2.0$, $P < 0.05$; Fig. 4], whereas a main effect of time was observed for saliva S-IgA secretion rate [$F(6,54) = 2.5$, $P < 0.05$; Fig. 4]. Saliva S-IgA concentration was significantly higher at 48 h on SDEP (40%; $P < 0.01$), and at 24 h (34%; $P < 0.05$) and 48 h (44%; $P < 0.01$) on SDEP + ER compared with 0 h. Saliva S-IgA concentration was significantly higher on SDEP and SDEP + ER at 24 h ($P < 0.05$), 48 h ($P < 0.01$) and pre-CAT ($P < 0.05$) compared with CON. Furthermore, a trend ($P = 0.07$) was apparent for saliva flow rate whereby a progressive decrease in saliva flow rate was observed by 48 h on SDEP (38%) and SDEP + ER (50%; Fig. 4). The CAT induced significant decreases in saliva S-IgA concentration on SDEP and SDEP + ER post-CAT ($P < 0.01$), 1-h and 2-h post-CAT ($P < 0.05$) compared with pre-CAT values. Additionally, saliva S-IgA secretion rate significantly decreased post-CAT compared with pre-CAT (24%; main effect of time $P < 0.05$). Both saliva S-IgA concentration (40%) and secretion rate (35%) were significantly lower post-CAT compared with 0-h values (main effect of time $P < 0.05$).

Plasma glucose, cortisol, epinephrine and norepinephrine

There were no trial × time interactions, but a main effect of time was observed for plasma glucose [$F(2,27) = 58.2$, $P < 0.01$], cortisol [$F(2,27) = 8.9$, $P < 0.01$] and norepinephrine concentration [$F(4,36) = 65.7$, $P < 0.01$; Table 2].

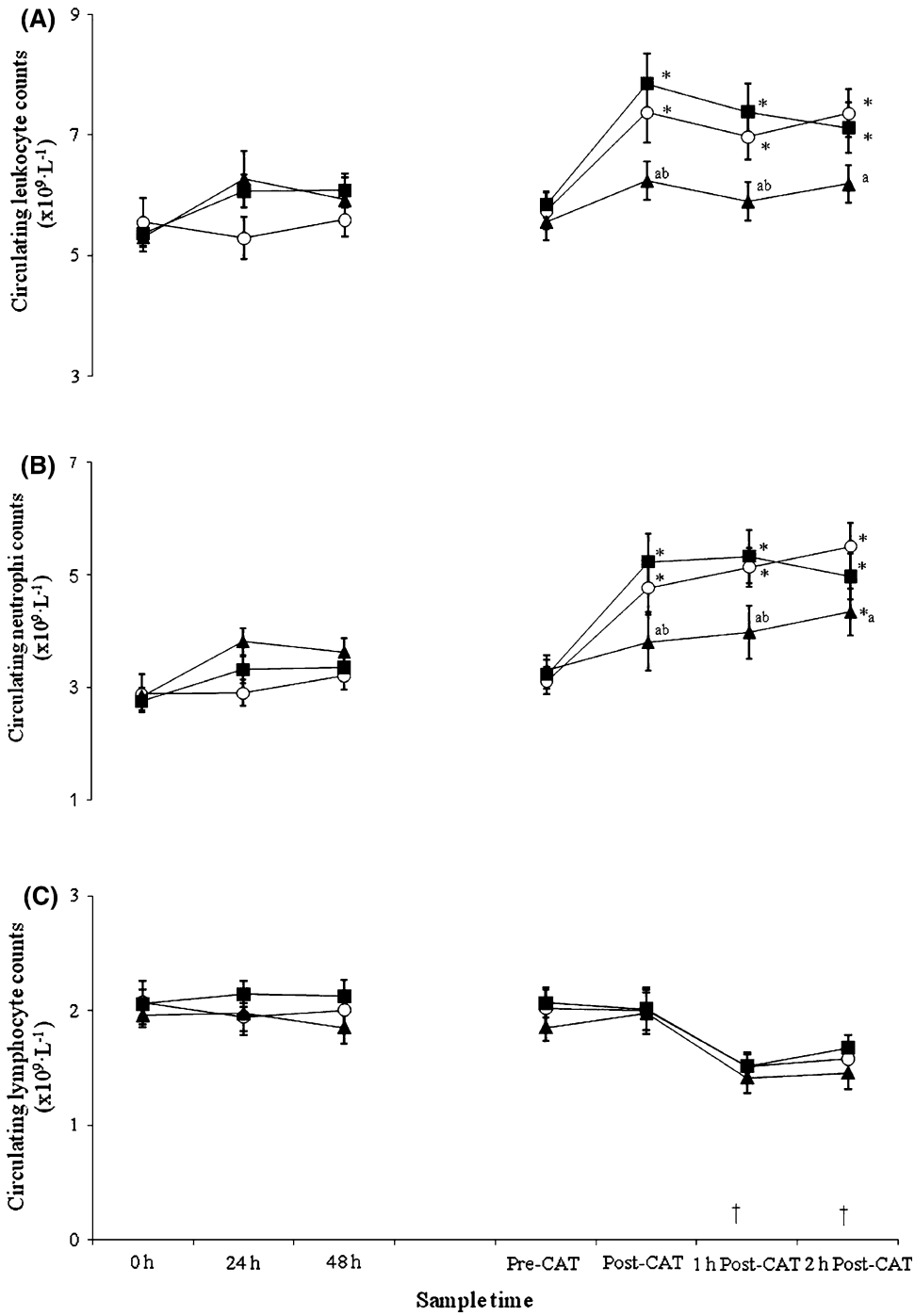

Fig. 2 Circulating counts of leucocytes (**a**), neutrophils (**b**) and lymphocytes (**c**) in response to a cold air test (CAT) after a 53-h period of normal sleep (CON, *open circles*), total sleep-deprivation (SDEP, *filled squares*) and total sleep-deprivation and 90% energy restriction (SDEP + ER, *filled triangles*). Mean (SEM) ($n = 10$). †Main effect of time $P < 0.05$ versus pre-CAT; *$P < 0.05$ versus pre-CAT; [a]$P < 0.05$ versus CON; [b]$P < 0.05$ versus SDEP

No main effects were observed for plasma epinephrine concentration. Plasma glucose concentration significantly increased 2-h post-CAT after the standardised meal ($P < 0.01$ vs. pre-CAT), whereas plasma cortisol (31%) and norepinephrine (346%) concentrations significantly increased post-CAT ($P < 0.05$ and $P < 0.01$, respectively vs. pre-CAT). Plasma cortisol concentration returned to pre-CAT levels 1 h into recovery, while plasma norepinephrine concentration remained significantly above pre-CAT levels 1 h into recovery ($P < 0.05$). Furthermore, a trend ($P = 0.09$) was apparent for plasma norepinephrine concentration whereby SDEP + ER induced a higher norepinephrine response post-CAT and during recovery compared with SDEP and CON.

Discussion

The aims of the current study were threefold. The first aim was to determine the effects of two nights of sleep

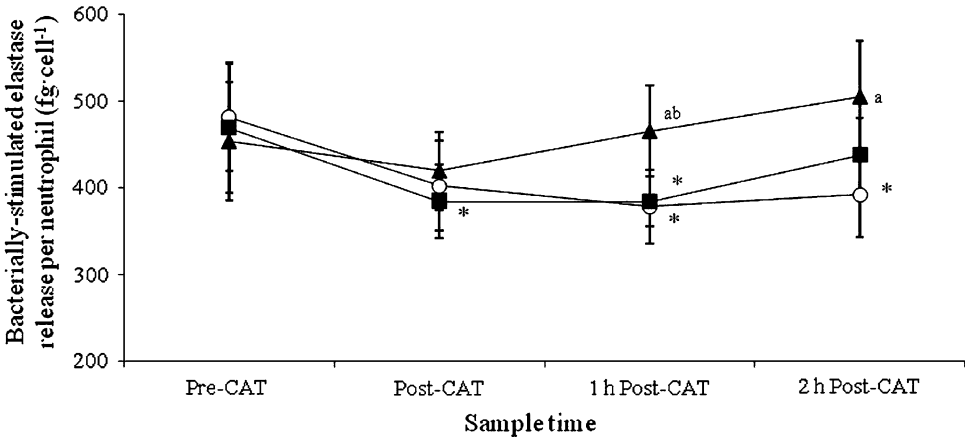

Fig. 3 Bacterially stimulated elastase release per neutrophil response to a cold air test (CAT) after a 53-h period of normal sleep (CON, *open circles*), total sleep-deprivation (SDEP, *filled squares*) and total sleep-deprivation and 90% energy restriction (SDEP + ER, *filled triangles*). Mean (SEM) ($n = 10$). *$P < 0.05$ versus pre-CAT; [a]$P < 0.05$ versus CON; [b]$P < 0.05$ versus SDEP

deprivation with and without energy restriction on selected immune indices at rest. The second aim was to determine the effects of passive cold exposure on selected immune indices. The third aim was to determine the effects of sleep deprivation with and without energy restriction on the response of immune indices to subsequent passive cold exposure. Compared with multi-stressor studies in the field, a particular strength of the present study is that the experimental procedures were carried out under controlled laboratory conditions enabling us to better determine the influence of sleep deprivation alone or sleep deprivation with energy restriction on immune indices. In addition, by removing participants from the CAT at a pre-defined body core temperature (T_{re} 36°C), rather than after a pre-defined time period, we were able to more accurately assess the influence of modest whole-body cooling on immune indices. In contrast to two of our hypotheses, two nights of sleep deprivation with and without energy restriction did not compromise immune indices at rest or amplify responses to subsequent modest whole-body cooling. In support of one of our hypotheses, modest whole-body cooling decreased circulating lymphocytes, bacterially stimulated neutrophil degranulation and saliva S-IgA responses. These data suggest that modest whole-body cooling poses a greater threat to immune status than two nights of sleep deprivation alone or two nights of sleep deprivation in combination with 90% energy restriction.

In the current study, two nights of sleep deprivation alone or with 90% energy restriction failed to induce significant alterations in resting circulating leucocytes. These findings extend those of a recent study showing that one night of sleep deprivation did not alter resting circulating leucocyte or differential leucocyte counts (Costa et al. 2009). On the contrary, we recently showed that 48 h of 90% energy restriction alone decreased circulating leucocyte counts mainly due to a decrease in circulating lymphocyte counts (Laing et al. 2008b). This discrepancy highlights that an individual stressor known to decrease aspects of immune function (e.g. energy restriction) does not necessarily have the same effect when combined with another stressor (e.g. sleep deprivation). It remains unclear why the addition of sleep deprivation to energy restriction prevented the decrease in circulating lymphocytes shown previously (Laing et al. 2008b). Possibilities include the influence of sleep deprivation on circadian modulation of regional blood flow (Van Someren 2006) and the likely knock-on effects on lymphocyte redistribution (Kruger and Mooren 2007). Increases in circulating melatonin during sleep deprivation (Salin-Pascual et al. 1988) might modulate regional blood flow, and therefore account for an alteration in lymphocyte redistribution (Guerrero and Reiter 2002), but this remains unsubstantiated.

The relatively modest leucocytosis immediately post-CAT on SDEP and CON is likely explained by demargination of neutrophils from the vascular endothelium (McCarthy and Dale 1988). Although raised circulating norepinephrine post-CAT (Table 2) might play a role in demarginating neutrophils from the vascular endothelium, other mechanisms must be at work as we observed similar increases in circulating norepinephrine on all trials post-CAT, but a 2-h delay in neutrophilia when energy restriction was super-imposed onto sleep deprivation (SDEP + ER). This once again highlights the complex interaction of various stressors on immune indices. Cold exposure is widely acknowledged to increase plasma norepinephrine due to SAM axis stimulation which in-turn promotes vasoconstriction, shivering thermogenesis and endogenous energy substrate breakdown (Castellani et al. 2002). Raised circulating norepinephrine might account for the lymphopenia during recovery after the CAT possibly via alterations in adhesion molecules (e.g. CD 62L), and surface receptors (e.g. CXCR4) on lymphocytes thought to have a key role in governing lymphocyte trafficking during environmental stress (Kruger and Mooren 2007). The small but significant increase in plasma cortisol from pre-CAT (12 noon) to post-CAT is unlikely to account for the

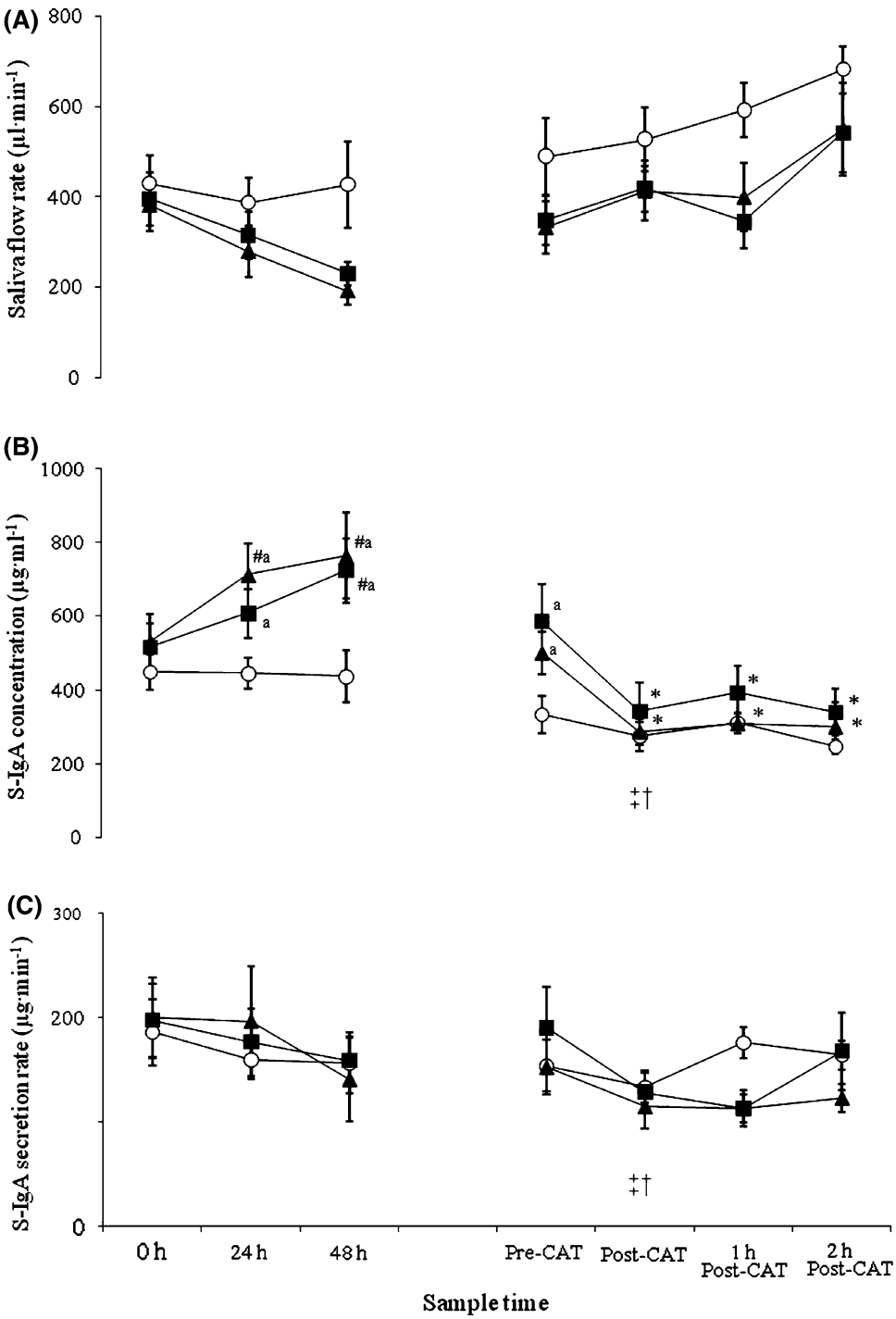

Fig. 4 Saliva flow rate (**a**), S-IgA concentration (**b**) and S-IgA secretion rate (**c**) responses to a cold air test (CAT) after a 53-h period of normal sleep (CON, *open circles*), total sleep-deprivation (SDEP, *filled squares*) and total sleep-deprivation and 90% energy restriction (SDEP + ER, *filled triangles*). Mean (SEM) ($n = 10$). ‡Main effect of time $P < 0.05$ versus 0 h; †Main effect of time $P < 0.05$ versus pre-CAT; #$P < 0.05$ versus 0 h; *$P < 0.05$ versus pre-CAT; [a]$P < 0.05$ versus CON

observed changes in leucocyte trafficking because post-CAT plasma cortisol was similar to 0 h (0800 h). In addition, substantial biological effects of cortisol tend to be reported only when the plasma cortisol concentration exceeds the capacity of the corticosteroid-binding globulin (550 nmol l^{-1}) and free cortisol concentration increases (McCarthy and Dale 1988).

Two nights of sleep deprivation alone or with 90% energy restriction did not compromise bacterially stimulated neutrophil degranulation at rest. This finding extends those recently showing that one night of sleep deprivation alone (Costa et al. 2009) and 2 days of 90% energy restriction alone did not alter resting neutrophil degranulation (Laing et al. 2008b). However, here we have also shown under controlled laboratory conditions that modest whole-body cooling (T_{re} 35.9°C) decreases neutrophil degranulation. It is possible that the decrease in neutrophil degranulation after the CAT on SDEP and CON

Table 2 Plasma glucose, cortisol, epinephrine and norepinephrine responses ($n = 10$) to a cold air test (CAT) after a 53-h period of normal sleep (CON), total sleep-deprivation (SDEP), and total sleep-deprivation and 90% energy-restriction (SDEP + ER)

	0 h	24 h	48 h	Pre-CAT	Post-CAT	1-h post-CAT	2-h post-CAT
Glucose (mmol l^{-1})							†
CON	4.7 (1.0)	4.9 (0.8)	4.9 (0.8)	4.9 (1.1)	5.1 (1.1)	5.0 (1.6)	7.5 (2.4)
SDEP	4.6 (1.1)	4.7 (1.0)	5.0 (0.8)	4.6 (0.9)	4.8 (1.3)	5.1 (1.0)	7.7 (1.5)
SDEP + ER	4.6 (1.3)	4.2 (1.3)	4.6 (0.8)	4.3 (1.2)	4.7 (0.9)	4.5 (1.0)	7.0 (1.7)
Cortisol (nmol l^{-1})				‡	†	‡	‡
CON	468 (181)	507 (171)	550 (160)	351 (96)	450 (175)	256 (101)	286 (105)
SDEP	491 (168)	497 (184)	535 (210)	320 (90)	392 (181)	261 (104)	258 (85)
SDEP + ER	487 (169)	584 (215)	552 (191)	291 (79)	421 (186)	305 (121)	355 (117)
Epinephrine (nmol l^{-1})							
CON	0.4 (0.2)		0.2 (0.1)	0.3 (0.1)	0.4 (0.2)	0.3 (0.1)	
SDEP	0.3 (0.2)		0.3 (0.2)	0.4 (0.2)	0.4 (0.1)	0.3 (0.2)	
SDEP + ER	0.4 (0.2)		0.3 (0.2)	0.4 (0.3)	0.5 (0.3)	0.3 (0.2)	
Norepinephrine (nmol l^{-1})					†	†	
CON	1.2 (0.5)		1.5 (0.4)	1.7 (0.7)	7.4 (2.6)	3.1 (0.7)	
SDEP	1.2 (0.3)		1.6 (0.5)	1.7 (0.7)	8.2 (3.5)	3.5 (1.1)	
SDEP + ER	1.1 (0.3)		1.9 (0.7)	2.3 (0.8)	9.8 (3.3)	4.6 (1.4)	

Mean (SD)

‡ Main effect of time $P < 0.05$ versus 0 h

† Main effect of time $P < 0.05$ versus pre-CAT

can be explained by the demargination of intravascular neutrophils of different maturity status to those already in circulation and that these demarginated neutrophils responded less to bacterial challenge (Laing et al. 2005). That there was a significant neutrophilia on SDEP and CON immediately after the CAT but not on SDEP + ER when there was no decrease in neutrophil degranulation lends support to this possibility. Put another way, the lack of a CAT-evoked decrease in neutrophil degranulation on SDEP + ER probably reflects the similar population of neutrophils in circulation before and after the CAT on this trial. It is noteworthy though that the mediators of the neutrophilia and decrease in neutrophil degranulation in response to stressors (e.g. exercise) are not as clear-cut as previously thought and require further clarification (Laing et al. 2008a). For example, responses of the widely accepted mediators of the neutrophilia to acute stress (e.g. circulating catecholamines and cortisol) in the present study do not provide adequate explanations for our observations. In short, we observed no difference between trials in CAT-evoked responses of circulating catecholamines and cortisol in the presence of an immediate neutrophilia post-CAT on SDEP and CON but a 2 h delayed neutrophilia on SDEP + ER.

Decreases in saliva flow rate and saliva IgA have been associated with a higher incidence of respiratory tract infections (Gleeson 2000). Two nights of sleep deprivation alone or with 90% energy restriction did not compromise mucosal immunity at rest in the present study. These findings extend those recently showing that one night of sleep deprivation alone (Costa et al. 2009) and 2 days of 90% energy restriction alone (Oliver et al. 2007) do not lower resting saliva IgA concentration or secretion rate. However, the current study shows that two nights of sleep deprivation alone or with 90% energy restriction tended to decrease saliva flow rate ($P = 0.07$) with concomitant significant increases in saliva S-IgA concentration. The increase in saliva S-IgA concentration on SDEP and SDEP + ER likely reflects a concentrating effect due to the trend for a decrease in saliva flow rate. The trend for the decrease in saliva flow rate during sleep deprivation trials probably results from a decrease in parasympathetic tone due to the anxiety associated with prolonged sleep deprivation (Proctor and Carpenter 2007).

It is quite conceivable that other stressors such as heavy physical exertion and dehydration contribute to the decrease in saliva IgA responses reported in multi-stressor field studies (Gleeson 2000; Gomez-Merino et al. 2003; Oliver et al. 2007; Walsh et al. 2004). Little is currently known about the effects of cold exposure that evokes a decrease in body core temperature on saliva S-IgA responses (Walsh and Whitham 2006). Studies have examined saliva IgA responses to prolonged exercise in cold conditions, but body core temperature did not fall in those studies (Tomasi et al. 1982; Walsh et al. 2002). A novel finding in the current study is that modest

whole-body cooling (T_{re} 35.9°C) was associated with a decrease in saliva S-IgA responses. The time course of the response indicates that modest whole-body cooling decreased S-IgA translocation as synthesis of S-IgA takes many hours to days (Hucklebridge et al. 1998). Plausible explanations for the decrease in saliva S-IgA we observed with modest whole-body cooling include the effects of cold air decreasing the temperature of the mucosal membranes, a possible drying effect of cold air and whole-body cooling-evoked neuro-endocrine regulation of trans-epithelial S-IgA translocation (Giesbrecht 1995; Proctor and Carpenter 2007). With an appropriate experimental design, a future study could delineate the effects of breathing cold air from the effects of a decrease in body core temperature on saliva S-IgA responses.

In conclusion, two nights of sleep deprivation with and without energy restriction did not compromise immune indices at rest. However, modest whole-body cooling alone (T_{re} 35.9°C) decreased circulating lymphocytes, neutrophil degranulation and S-IgA responses, but these responses were not amplified by prior sleep deprivation alone or sleep deprivation with energy restriction. Future studies should assess immune responses, including where possible in vivo immune responses, in clinically hypothermic individuals ($T_{re} \leq 35$°C) and the clinical significance of any reductions in immune function for infection incidence and tissue healing (e.g. from frostbite and non-freezing cold injury).

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